OECD Environment, Health and Safety Publications

Series on Testing and Assessment

No. XX

DRAFT GUIDANCE DOCUMENT ON USING CYTOTOXICITY TESTS TO ESTIMATE STARTING DOSES FOR ACUTE ORAL SYSTEMIC TOXICITY TESTS

IOMC
INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
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399 **PREAMBLE**
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401 1. To be added
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402

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459

460 **INTRODUCTION**

461 1. The idea that *in vitro* cytotoxicity data could be used to determine the starting doses for rodent
462 acute oral toxicity tests, and subsequently reduce the number of animals used, was first discussed at a
463 workshop organized to evaluate the use of *in vitro* data for the classification and labeling of chemicals
464 (Seibert et al., 1996). The concept was later discussed and evaluated, along with a number of other
465 international initiatives, at a 2000 International Workshop on *In Vitro* Methods for Assessing Acute
466 Systemic Toxicity (ICCVAM, 2001). The approach was considered worthy of a formal validation study
467 to further investigate the linear relationship observed between IC_{50} values from *in vitro* basal cytotoxicity
468 tests and published rodent (rat and mouse) oral LD_{50} values from 347 chemicals in a Registry of
469 Cytotoxicity (RC) (Halle, 1998, 2003). The RC contains acute oral LD_{50} values for rats and mice obtained
470 from the Registry of Toxic Effects of Chemical Substances (RTECS®, Symyx Technologies, Inc.
471 Sunnyvale, CA, USA. http://www.symyx.com/products/databases/bioactivity/rtecs/index.jsp) and
472 published IC_{50} values for a variety of cytotoxicity endpoints and cell lines for the 347 chemicals with
473 known molecular weights.

474 2. To investigate the usefulness and limitations of standardized cytotoxicity tests for estimating
475 LD_{50} values, the National Toxicology Program [NTP] Interagency Center for the Evaluation of
476 Alternative Toxicological Methods [NICEATM] and the European Centre for the Validation of
477 Alternative Methods [ECVAM] sponsored and organized an international validation study using 72 coded
478 substances tested in three laboratories (ICCVAM, 2006a). Based on the results of the validation study on
479 cytotoxicity assays using BALB/c 3T3 mouse fibroblasts (3T3) and normal human epidermal
480 keratinocytes (NHK) in neutral red uptake (NRU), these test methods are recommended for determining
481 starting doses for acute oral toxicity tests using rats (ICCVAM, 2006a, b). The results of the study were
482 evaluated by an independent scientific peer review panel, which concluded that the methods were
483 adequately reliable and reproducible for use in a weight-of-evidence approach for determining starting
484 doses for acute oral toxicity tests (ICCVAM, 2006b). (Definitions used in the context of this Guideline
485 are set out in Annex 1.)

486 **INITIAL CONSIDERATION**

487 **Background Information**

488 3. The NRU *in vitro* basal cytotoxicity assay procedure is based on the ability of viable cells to
489 incorporate and bind neutral red (NR), a supravital dye (Borenfreund and Puerner, 1985). NR is a weak
490 cationic dye that readily diffuses through the plasma membrane and concentrates in lysosomes where it
491 electrostatically binds to the anionic lysosomal matrix. Toxicants can alter the cell surface or the
492 lysosomal membrane to cause lysosomal fragility and other adverse changes that gradually become
493 irreversible. Such adverse changes cause cell death and/or inhibition of cell growth, which then decrease
494 the amount of NR retained by the culture. Since the concentration of NR dye desorbed from the cultured
495 cells is directly proportional to the number of living cells, cytotoxicity is expressed as a concentration
496 dependent reduction of the uptake of NR after chemical exposure. The NRU assay uses a 96-well plate
497 format for the production of replicate measurements at eight test substance concentrations.

498 4. Data from the *in vitro* tests can be used for estimating the starting dose for acute oral systemic
499 toxicity tests. The *in vivo* starting dose is an estimated LD_{50} value calculated by inserting the *in vitro* IC_{50}
500 value into a regression formula derived from 282 substances for which there are both historical rat oral
501 LD_{50} values and *in vitro* IC_{50} values from the RC (ICCVAM, 2006a). For the 72 chemicals tested in the
502 NICEATM/ECVAM *in vitro* basal cytotoxicity validation study, inter-laboratory reproducibility of the
503 IC_{50}, measured by the average coefficient of variation (CV), was 47% for the 3T3 NRU assay and 28%
504 for the NHK NRU assay. Computer-simulated acute oral toxicity testing of the test substances indicated

that the animal savings were similar using either the 3T3 or the NHK NRU assays to determine starting doses (ICCVAM, 2006a).

5. Animal savings were highest for chemicals with LD_{50} >5000 mg/kg. For these less toxic chemicals, average animal use for the Up-and-Down Procedure (UDP; OECD, 2008) was reduced by up to 22% per test and average animal use for the Acute Toxic Class (ATC; OECD, 2001) method was reduced by up to 28% per test. An animal savings of up to 50% is possible using the cytotoxicity approach to a starting dose, compared to the number of animals used with the default starting dose in the UDP. Average animal use for the UDP or ATC method was reduced by 7% per test for the 72 substances used in the validation study, which were distributed across the five Globally Harmonized System of Classification and Labelling of Chemicals (GHS; UN, 2007) hazard categories and the unclassified category (>5000 mg/kg). However, a review of toxicity values in the European Union reveals that the majority of industrial substances tested for regulatory purposes have an LD_{50} of >2000 mg/kg. Eighty-seven percent of the chemicals in the New Chemicals Database (NCD), maintained at the Institute for Health and Consumer Protection (IHCP, DG-JRC, Ispra [http://ecb.jrc.it]), have LD_{50} >2000 mg/kg (Bulgheroni et al., 2009).

PRINCIPLE OF THE TEST METHOD

6. This Guidance Document describes methods to determine the *in vitro* basal cytotoxicity of test substances using NRU assays and then using the *in vitro* data generated to determine starting doses for *in vivo* acute oral systemic toxicity tests. The NRU assay is performed in a dose-response format to determine the concentration that reduces NRU by 50% compared to the controls (i.e., the IC_{50}). The IC_{50} value is used in a linear regression equation to estimate the oral LD_{50} value (dose that produces lethality in 50% of the animals tested), which is then used to determine a starting dose for acute oral toxicity testing using rats for the UDP or the ATC method. The use of the NRU test method to determine starting doses for these acute oral toxicity tests might reduce the number of animals required for the tests, and for relatively toxic substances, might reduce the number of animals that die or require humane euthanasia due to severe toxicity. The inter-laboratory validation study (ICCVAM, 2006a, b, c) demonstrated that the two test methods are useful and reproducible for this purpose. Standardized test method protocols (Stokes et al., 2008) provide details for performing NRU tests with rodent or human cells.

7. The NRU *in vitro* basal cytotoxicity assay involves exposing cells in culture to a test substance for 48 hours. The test substance is rinsed off the cells and the cells are then incubated with NR dye. The concentration of NR dye eluted from the cells is then quantitated spectrophotometrically. Stokes et al. (2008) describes the methods for testing substances using the immortalized rodent cell line, BALB/c 3T3 mouse fibroblasts (3T3), and primary human cells, normal human epidermal keratinocytes (NHK), in the NRU assay. The results for the two cell types proved to be similar in the validation study; however, the 3T3 NRU assay is more cost- and time-effective than the NHK NRU assay. Methods for preparation and dilution of substances to be tested in the *in vitro* NRU tests are also described along with a tiered solubility procedure to determine the best solvent for testing the substance of interest. Because the NHK NRU assay requires special attention concerning the cell culture medium, a medium pre-qualification procedure is provided (Annex 2).

DESCRIPTION OF THE TEST METHODS

Testing Formats

Range finder test

8. This is the initial cytotoxicity test performed to determine the starting doses for the main test. The NRU assays test eight concentrations of the test substance or the positive control (PC) by diluting the stock test substance solution in log dilutions to cover a large concentration range (see paragraphs 24-29).

Main test

9. The main test of the cytotoxicity assays is performed to determine the IC_{50} value (test substance concentration producing 50% inhibition of the endpoint measured, i.e., cell viability [see Annex 3]). The concentration closest to the range finder test IC_{50} value serves as the midpoint of the concentrations tested in the main test. Compared to the range finder test, the main test uses a smaller dilution factor for the concentrations tested (see paragraph 30).

Preparations for the 3T3 NRU Assay

Cells

10. The permanent murine fibroblast cell line, BALB/c 3T3 cells, clone 31, should be obtained from well qualified national/international cell culture repositories (e.g., American Type Culture Collection [ATCC], Manassas, VA, product # CCL-163 [http://www.atcc.org/]; the Health Protection Agency Culture Collections, Salisbury, UK [http://www.hpacultures.org.uk]; Japan Health Sciences Foundation, Health Science Research Resources Bank [HSRRB], National Institute of Biomedical Innovation, Osaka, Japan [http://www.jhsf.or.jp/English/index_gc.html]).

11. All cell stock and cultures used for testing should be certified as free of mycoplasma and bacterial contamination and should be checked frequently.

Media and culture conditions

12. Routine cell passage for the BALB/c 3T3 cells should use a culture medium containing Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with non-heat-inactivated 10% newborn calf serum (NCS) and 4 mM L-Glutamine and cells should be incubated at 37°C ±1°C, 90% ±5% humidity, and 5.0% ±1.0% CO_2/air. Cell culture conditions should assure that the cell cycle time is within the historical range of the cell line.

Preparation of cultures

13. The 3T3 cells from cryogenically-preserved stock should be subcultured at least twice before using the cells in the 3T3 NRU assay. Remove cells from flasks through trypsinization when cells reach 50% to 80% confluence. The passages of 3T3 cells from frozen stock should be limited to approximately 18 passages to avoid phenotypic and genotypic changes that may occur as the culture ages.

14. Cells in routine culture medium should be plated into 96-well tissue culture microtiter plates at a density of 2.0 – 3.0 x 10^3 cells/well (Annex 4 (Preferably the annexes should follow in the order they are cited in the text!)). Incubate cells for 24 hours ±2 hours to form a less than half (< 50%) confluent monolayer. This incubation period assures adequate cell recovery and adherence to allow for progression to the exponential growth phase.

Preparations for the NHK NRU Assay

Cells

15. Primary, non-transformed normal NHK can be substituted for the BALB/c 3T3 cells for the cytotoxicity assay. The NHK cells should come from cryopreserved primary or secondary pooled neonatal foreskin cells-procured only through commercial sources rather than preparing a primary culture from donated tissues (e.g., Clonetics #CC-2507 NHEK-Neonatal Normal Human Epidermal Keratinocytes, Pooled or equivalent [Lonza Walkersville, Inc., 8830 Biggs Ford Road, Walkersville, MD; https://bcprd.lonza.com/shop/b2c/start/(xcm=lonza_b2b&carea=DCEA16F3E87D10F18C7C001A4B525E10)/.do]).

16. All cell stock and cultures used for testing should be certified as free of mycoplasma and bacterial contamination and should be checked frequently.

Media and culture conditions

17. Routine cell passage for the NHK cells should include a serum-free defined keratinocyte basal culture medium supplemented with 0.0001 ng/mL human recombinant epidermal growth factor, 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 30 μg/mL gentamicin, 15 ng/mL amphotericin B, 0.10 mM calcium, and 30 μg/mL bovine pituitary extract (e.g., KBM® [Clonetics CC-3104], KBM® SingleQuots® [Clonetics CC-4131], and Clonetics Calcium SingleQuots® [CC-4202]; Lonza Walkersville, Inc., https://bcprd.lonza.com/shop/b2c/start/(xcm=lonza_b2b&carea=DCEA16F3E87D10F18C7C001A4B525E10)/.do). Cells should be incubated at 37°C ±1°C, 90% ±5% humidity, and 5.0% ±1.0% CO_2/air. Cell culture conditions should assure that the cell cycle time is within the historical range of the cell type.

Preparation of cultures

18. Propagate NHK cells (from cryopreserved pool) in 25 cm^2 tissue culture flasks. When cells reach 50% to 80% confluence, remove cells from flasks through trypsinization.

19. Prepare a cell suspension of $1.6 – 2.0x10^4$ cells/mL in NHK routine culture medium. Dispense 125 μL of the cell suspension ($2.0 – 2.5x10^3$ cells/well) to the test wells of a 96-well tissue culture microtiter plate (Annex 4). Dispense 125 μL routine culture medium into the peripheral blank wells.

20. Incubate cells for 48 – 72 hours (37ºC ±1ºC, 90% ±10% humidity, 5.0% ±1.0% CO_2/air) so that cells form a >20% confluent monolayer. This incubation period assures adequate cell recovery and adherence to allow for progression to the exponential growth phase.

<u>Preparation of Test Substance</u>

Test substances in solution

21. Equilibrate test substances to room temperature before dissolving and diluting. Prepare the test substance immediately prior to use rather than preparing in bulk for use in subsequent tests. The solutions should be clear and have no noticeable precipitate. Prepare at least 1-2 mL total volume of each stock dilution to ensure an adequate quantity for all of the test wells in a single 96-well plate. Preparation of test substances under red or yellow light is recommended to preserve substances that degrade upon exposure to light.

22. For substances dissolved in dimethyl sulfoxide (DMSO) or ethanol (ETOH), the final DMSO or ETOH concentration for application to the cells should be no more than 0.5% (v/v) in the VCs and in all of the eight test concentrations. The concentration of DMSO or ETOH should be the minimum concentration needed to dissolve the test substance.

23. Prepare the stock solution for each test substance at the highest concentration found to be soluble in the solubility test (Annex 4). The highest test concentration applied to the cells in a range finding test is as follows:

- 0.5 times the highest concentration found to be soluble in the solubility test, if the substance was soluble in culture medium, or
- 1/200 the highest concentration found to be soluble in the solubility test if the substance was soluble in DMSO or ETOH.

Preparation of test substance in solvent using a log dilution scheme

24. This log dilution scheme is appropriate for preparing test substances for the range finder test (see paragraph 8).

25. Dissolve the test substance in DMSO or ETOH at 200 mg/mL to prepare the test substance stock solution. Prepare the seven lower concentrations by successive serial dilutions that decrease by one log unit each (e.g., 0.1 mL of solution into 0.9 mL solvent).

26. Each concentration is 200 fold greater than the concentration to be tested. Make a 1:100 dilution by diluting one part dissolved test substance in each tube with 99 parts of medium (e.g., 0.1 mL test substance in DMSO or ETOH + 9.9 mL medium) to derive the eight 2X concentrations for application to the cells. Each 2X test substance concentration will then contain 1% (v/v) solvent.

27. The 3T3 cells will have 50 µL Routine Culture Medium in the wells prior to application of the test substance. Adding 50 µL of any specific 2X test substance concentration to the assigned wells will appropriately dilute the test substance (e.g., highest concentration in well will be 1,000 µg/mL) in 100 µL and the solvent concentration in the wells will be 0.5% (v/v).

28. The NHK cells will have 125 µL of culture medium in the wells prior to application of the test substance. Adding 125 µL of any specific 2X test substance concentration to the assigned wells will appropriately dilute the test substance (e.g., highest concentration in well will be 1,000 µg/mL) in 250 µL and the solvent concentration in the wells will be 0.5% (v/v).

29. A test substance prepared in medium or solvent may precipitate upon transfer into the Routine Culture Medium.

Test substance dilutions

30. The main test (see paragraph 9) requires a smaller dilution factor than the range finder test. The dilution factor of 3.16 (= $\sqrt[2]{10}$) divides a log into two equidistant steps, 2.15 (= $\sqrt[3]{10}$) into three steps, 1.78 (= $\sqrt[4]{10}$) into four steps, 1.47 (= $\sqrt[6]{10}$) into six steps, and 1.21 (= $\sqrt[12]{10}$) into 12 steps (see Table 1). For example, to make dilutions with the dilution factor of 1.47: Dilute 1 volume of the highest concentration by adding 0.47 volumes of diluent. After equilibration, dilute 1 volume of this solution by adding 0.47 volumes of diluent...(etc.).

Table 1 Maximum Doses for Test Substances Prepared in Routine Culture Medium for the Main Test

Number of Equal Dilutions	Concentration Units												
2	10						31.6						100
3	10				21.5				46.4				100
4	10			17.8				31.7			56.4		100
6	10		14.7		21.5		31.6		46.4		68.1		100
12	10	12.1	14.7	17.8	21.5	26.1	31.6	38.3	46.4	56.2	68.1	82.5	100

31. The highest test substance concentration that may be applied to the cells in the main tests will be either 100 mg/mL, or the maximum soluble dose. If minimal or no cytotoxicity was measured in the range finder test, the maximum dose for the main tests is established as follows:

a) Weigh the test substance into a glass tube and add routine culture medium to obtain a concentration of 200 mg/mL. Mix the solution using the mixing procedures that produced solubility when performing the solubility test (Annex 5).

b) If complete solubility is achieved in medium, then prepare seven additional serial stock dosing solutions from the 200 mg/mL 2X stock.

c) If the test substance is insoluble in medium at 200 mg/mL, proceed by adding medium, in small incremental amounts, to attempt to dissolve the substance by using the sequence of mixing procedures specified in Annex 5. More stringent solubility procedures may be employed if needed based on results from the range finder test.

d) Use the highest soluble stock solution to prepare the seven additional serial stock dosing solutions.

Maximum doses for test substances prepared in DMSO or ETOH for the main test

32. The highest test substance concentration that may be applied to the cells in the main tests will be ≤ 2.5 mg/mL or less, depending upon the maximum solubility in solvent.

a) Weigh the test substance into a glass tube and add the appropriate solvent (determined from the original solubility test [Annex 5]) to obtain a concentration of 500 mg/mL. Mix the test substance solution using the sequence of mixing procedures specified in Annex 5. If complete solubility is achieved in the solvent, then prepare seven additional serial stock dosing solutions from the 500 mg/mL 200X stock.

b) If the test substance is insoluble in solvent at 500 mg/mL, proceed by adding solvent, in small incremental amounts, to attempt to dissolve the substance by again using the sequence of mixing procedures.

c) Use the highest soluble stock solution to prepare the seven additional serial stock dosing solutions. If precipitates are observed in the 2X dilutions, continue with the test and make the appropriate observations and documentation.

Test Conditions

Test substance concentrations

Controls

33. *Positive Control (PC)*: Sodium lauryl sulfate (SLS; CASRN 151-21-3). Prepare a separate 96-well plate of eight PC concentrations so that a complete dose-response curve (Annex 3), rather than a single point estimate, can be obtained. This will assist with troubleshooting the test (Annex 6), if the need arises. Multiple test substance plates can be run with a single PC plate. The PC plate will follow the same schedule and procedures used for the test substance plates.

34. *Vehicle Control (VC)*: The VC consists of routine culture medium when the test substances are dissolved in culture medium. For test substances dissolved in the solvents DMSO or ETOH, the VC consists of routine culture medium with the same amount of solvent (0.5% [v/v]) as is applied to the 96-well test plate.

Test Procedure

Range finder test

35. Test eight concentrations (see paragraph 25) of the test substance by diluting the stock solution using log dilutions (e.g., 1:10, 1:100, 1:1000). If a range finder test does not generate adequate cytotoxicity for the calculation of an IC_{50} value, then higher doses should be attempted. If cytotoxicity is limited by solubility, then more stringent solubility procedures to increase the stock concentration (Annex 5) should be employed.

Main test

36. Use the range finder IC_{50} value as a central concentration and adjust dilutions higher and lower in equal steps. Alternatively, the test substance concentration closest to the range finder IC_{50} value could be used as the central value.

37. Use a smaller dilution factor for the concentration series of the main test (e.g., dilution factor of $\sqrt[6]{10} = 1.47$) than that used for the range finder test. The slope of the range finder concentration-response can be used to approximate the dilution factor.

38. Cover the relevant concentration range around the IC_{50} (> 0% and < 100% effect), preferably with several points of a graded effect, but with a minimum of two points, one on each side of the IC_{50}, and avoid too many (e.g., > 6) concentrations on either end of the concentration spectrum.

39. Perform a minimum of two main tests for a test substance and average the IC_{50} results.

3T3 NRU Assay

Day 1

40. Prepare a cell suspension of $2.0 – 3.0 \times 10^4$ cells/mL in the routine culture medium and dispense 100 µL of the cell suspension to the test wells ($2.0 – 3.0 \times 10^3$ cells/well) of a 96-well tissue culture microtiter plate (Annex 3). Dispense 100 µL of the routine culture medium without cells into the peripheral blank wells of the test plate. Incubate cells for 24 hours ±2 hours to form a less than half (< 50%) confluent monolayer.

Day 2

41. Remove Routine Culture Medium from the cells after incubation period by careful inversion of the plate (i.e., dump). Gently blot the plate on a sterile paper towel to remove residual culture medium. Immediately add 50 µL of fresh pre-warmed (37ºC ±1ºC) routine culture medium to all wells. Add 50 µL of test substance in the test substance dilution medium (DMEM without serum, 4 mM L-Glutamine 200 IU/mL penicillin, 200 µg/mL streptomycin) and 50 µL of test substance dilution medium (for VCs) to the appropriate wells (Annex 4). Incubate cells for 48 hours ±0.5 hours.

Day 4

Microscopic Procedure

42. After at least 46 hours of treatment, examine each plate with a phase contrast microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells. Record any changes in morphology of the cells due to the cytotoxic effects of the test substance, but do not use these records for any quantitative measure of cytotoxicity. Undesirable growth characteristics of control cells may indicate experimental error and may be cause for rejection of the assay. Perform the NRU assay (see paragraphs 46-51).

NHK NRU Assay

Day 1

43. After the initial cell culture has reached 50% to 80% confluence, remove cells from flasks through trypsinization. Prepare a cell suspension of $1.6 – 2.0 \times 10^4$ cells/mL in NHK routine culture medium. Dispense 125 µL of the cell suspension ($2.0 – 2.5 \times 10^3$ cells/well) to the test wells of a 96-well tissue culture microtiter plate (Annex 4). Dispense 125 µL routine culture medium into the peripheral blank wells. Incubate cells for 48 – 72 hours (37ºC ±1ºC, 90% ±10% humidity, 5.0% ±1.0% CO_2/air) so that cells form a >20% confluent monolayer.

Day 3

44. After the incubation period, do not remove the NHK routine culture medium from the test plate. Add 125 µL of the appropriate concentration of test substance in routine culture medium (see paragraph 28) to the appropriate wells. Incubate cells for 48 hours ±0.5 hours.

Day 5

Microscopic Procedure

45. *(See paragraph 42, Day 4 3T3 NRU assay)*

Neutral Red Uptake Assay

46. After incubation, remove (i.e., dump) the medium from the wells and rinse the cells carefully with 250 µL/well pre-warmed Dulbecco's Phosphate Buffered Saline (D-PBS). Remove the rinsing solution by inversion of the plate and blot dry on paper towels.

47. *3T3 Cells*: Add 250 µL of 25 µg/mL NR dye in DMEM with 5% NCS, 4 mM L-Glutamine, 100 IU/mL Penicillin, and 100 µg/mL Streptomycin to all wells (including the blanks) and incubate at 37ºC ±1ºC, 90% ±10% humidity, 5.0% ±1.0% CO_2/air for 3.0 hours ±0.1 hr.

48. *NHK Cells*: Add 250 µL of 33 µg /mL Neutral Red (NR) dye in NHK routine culture medium to all wells (including the blanks) and incubate at 37ºC ±1ºC, 90% ±10% humidity, 5.0% ±1.0% CO_2/air for 3.0 hours ±0.1 hr.

49. After incubation remove the NR medium, and carefully rinse cells with 250 µL/well pre-warmed D-PBS. Remove the solution as above. Add 100 µL NR desorb solution (freshly prepared 49 parts water + 50 parts ethanol + 1 part glacial acetic acid) to all wells (including blanks) to extract the dye.

50. Shake the microtiter plates rapidly on a microtiter plate shaker for 20 – 45 minutes. Protect the plates from light while shaking. Plates should be still for at least five minutes after removal from the plate shaker/mixer. Rupture any bubbles prior to reading the plate.

51. Measure the light absorption (optical density [OD]) within 60 minutes of adding NR desorb solution of each well at 540 nm ±10 nm (OD_{540}) in a microtiter plate reader (spectrophotometer), using the blanks as a reference. Save the data in an appropriate electronic file format for subsequent analysis.

DATA AND REPORTING

Quality and Quantity of Data

Test acceptance criteria

52. The mean of the left (VC1) and the mean of the right (VC2) columns of VCs (see Annex 4) do not differ by more than 15% from the mean of all VCs.

53. At least one calculated cytotoxicity value > 0% and ≤ 50% viability and at least one calculated cytotoxicity value > 50% and < 100% viability should be present. Exception: If a test has only one point between 0 and 100% **and** the smallest practical dilution factor (i.e., 1.21) was used **and** all other test acceptance criteria were met, then the test is acceptable.

Additional test acceptance criteria for the PC

54. The PC dose-response should have an R^2 (coefficient of determination) ≥ 0.85 for the Hill model fit.

55. The PC IC_{50} value should be within ±2.5 standard deviations (SD) of the historical mean established by the laboratory.

Evaluation of Results

Anticipated results

56. For either NRU test, blank OD_{540} values should be approximately 0.05 (ICCVAM, 2006a). The corrected OD_{540} for the VCs can be expected to average 0.476 ±0.117 (SD) for the 3T3 NRU and 0.685 ±0.175 (SD) for the NHK NRU (ICCVAM, 2006a). IC_{50} values for the positive control, SLS, should be

41.5 ±4.8 (SD) µg/mL (n = 233) for the 3T3 NRU assay and 3.11 ±0.72 µg/mL (n = 114) for the NHK NRU assay. Annex 3 shows a typical dose-response curve for SLS in the 3T3 NRU assay. IC_{50} results for the test substances in the NICEATM/ECVAM *in vitro* basal cytotoxicity validation study ranged from 0.005 to 38,878 µg/mL (1.1×10^{-5} to 422 mM) for the 3T3 NRU test method and 0.00005 to 49,800 µg/mL (6.4×10^{-8} to 49,800 mM) for the NHK NRU test method (ICCVAM, 2006a).

Interpretation of Results

Determination of the starting doses for acute oral systemic toxicity tests (see Annex 7)

57. Use the IC_{50} value in mM in the following regression formula to estimate the log LD_{50} in mmol/kg:

$$\log LD_{50}\ (\text{mmol/kg}) = 0.439 \log IC_{50}\ (\text{mM}) + 0.621 \text{ (ICCVAM, 2006a).}$$

Convert the log LD_{50} to LD_{50} and then convert to mg/kg units by multiplying by the molecular weight of the test substance.

58. The starting dose for the UDP is the next dose lower than the estimated LD_{50} in the default dose progression. The default dose progression for the UDP is 1.75, 5.5, 17.5, 55, 175, 550, and 2000 mg/kg using a limit test of 2000 mg/kg or 1.75, 5.5, 17.5, 55, 175, 550, 1750, and 5000 mg/kg using a limit test of 5000 mg/kg (OECD, 2001a).

59. The starting dose for the ATC method is the next dose lower than the estimated LD_{50} in the default dose progression. The default dose progression for the ATC method is 5, 50, 300, or 2000 mg/kg for the 2000 mg/kg limit test or 5, 50, 300, 2000, or 5000 mg/kg for the 5000 mg/kg limit test.

60. For substances with no molecular weight, IC_{50} values in µg/mL can be used in the following regression formula to estimate the LD_{50} in mg/kg:

$$\log LD_{50}\ (\text{mg/kg}) = 0.372 \log IC_{50}\ (\mu\text{g/mL}) + 2.024 \text{ (ICCVAM, 2006a)}$$

Interpretation of Data

61. Use good biological/scientific judgment for determining *unusable* wells that will be excluded from the data analysis.

62. After subtraction of the blank OD_{540} value, calculate the cell viability for each test well as percent of the mean VC OD_{540} value. Cell viability can be calculated using a spreadsheet template (e.g., Microsoft Excel®). Ideally, the eight concentrations of each substance tested will span the range of no effect up to total inhibition of cell viability.

63. Perform a Hill function analysis of the replicate cell viability data for each concentration using statistical software (e.g., GraphPad PRISM®) to calculate the IC_{50} for each test substance. The Hill function is recommended because all the dose-response information, rather than a few points around the IC_{50}, is used. The Hill function also provides the slope of the dose-response curve (see Annex 1).

Test Report

64. The test report should contain the following test and test substance information:

Test and Control Substances

- chemical/substance name(s), synonyms, CASRN, formula weight, if known
- purity and composition of the substance or preparation (in percentage[s] by weight)
- physicochemical properties (e.g., physical state, volatility, pH, stability, chemical class, water solubility)

– solubilization of the test/control substances (e.g., vortexing, sonication, warming, grinding) prior to testing, if applicable

Solvent

– solvent name

– justification for choice of solvent

– solubility of the test substance in the solvent

– percentage of solvent in treatment medium and vehicle controls

Cells

– cell type used and source of cells

– absence of mycoplasma or bacterial contamination

– cell passage number, if known

Test Conditions (1); experimental information

– experiment start and completion dates

– details of test procedures used

– description of modifications made to the test procedure

– reference to historical data of the test model (e.g., solvent and PCs)

– description of the evaluation criteria used

Test Conditions (2); incubation before and after treatment

– composition of culture medium used for routine cell culture and test substance application

– incubation conditions (i.e., 37°C ±1°C, 90% ±5% humidity, and 5.0% ±1% CO_2/air)

– duration of incubation (pre-treatment; post-treatment)

Test Conditions (3); treatment with test substance

– rational for selection of concentrations of the test substance

– solubility of the test substance and rationale of the highest test concentration

– composition of the treatment medium

– duration of the test substance treatment

Test Conditions (4); Neutral Red viability test

– composition of Neutral Red treatment medium

– duration of Neutral Red incubation

– incubation conditions (i.e., 37°C ±1°C, 90% ±5% humidity, and 5.0% ±1.0% CO_2/air)

– Neutral Red extraction conditions (extractant; duration)

– wavelength used for spectrophotometric reading of Neutral Red optical density

Information Concerning the Sponsor and the Test Facility

– name and address of the sponsor, test facilities, study director, and participating laboratory technicians

868 – justification of the test method and specific protocol used

869 *Test Method Integrity*

870 – the procedure used to ensure the integrity (i.e., accuracy and reliability) of the test method over
871 time (e.g., use of the PC data)

872 *Criteria for an Acceptable Test*

873 – acceptable VC differences between each column of wells and the mean of both columns

874 – acceptable concurrent PC ranges based on historical data (include the summary historical data)

875 – number of toxic points on either side of the IC_{50} (i.e., number of points > 0 and
876 ≤ 50% viability and > 50 and < 100% viability)

877 *Results*

878 – tabulation of data from individual test samples (e.g., IC_{50} values for the reference substance and
879 the PC, absolute and derived OD_{540} readings, reported in tabular form, including data from replicate
880 repeat experiments as appropriate, and the means and standard deviations for each experiment)

881 *Description of Other Effects Observed*

882 – cell morphology, precipitate, NR crystals, etc.

883 *Discussion of the Results*

884 *Conclusions*

885 *Quality Assurance (QA) Statement for GLP-Compliant Studies*

886 – statement describing all inspections and other QA activities during the study, and the dates results
887 were reported to the Study Director; statement can confirm that the final report reflects the raw data

888

889

LITERATURE

(1) Borenfreund, E., and Puerner, J.A. 1985. Toxicity determination *in vitro* by morphological alterations and neutral red absorption. *Toxicology Letters* 24:119-124.

(2) Bulgheroni A, Kinsner-Ovaskainen A, Hoffmann S, Hartung T, Prieto P. 2009. Estimation of acute oral toxicity using the No Observed Adverse Effect Level (NOAEL) from the 28 day repeated dose toxicity studies in rats. *Regul Toxicol Pharmacol* Feb;53(1):16-9.

(3) EPA (US Environmental Protection Agency). 1996. Product Properties Test Guidelines. OPPTS 830.7840 Water Solubility: Column Elution Method; Shake Flask Method. Office of Prevention, Pesticides and Toxic Substances. EPA 712–C–98–041. Available: http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/830_Product_Properties_Test_Guidelines/Series/830-7840.pdf

(4) Halle, W. 1998. Toxizitätsprüfungen in Zellkulturen für eine Vorhersage der akuten Toxizität (LD_{50}) zur Einsparung von Tierversuchen. Life Sciences/Lebenswissenschaften, Volume 1, Jülich: Forschungszentrum Jülich.

(5) Halle, W. 2003. The Registry of Cytotoxicity: Toxicity testing in cell cultures to predict acute toxicity (LD50) and to reduce testing in animals. *Alternatives to Laboratory Animals* 31:89-198. (*English translation of Halle 1998*).

(6) ICCVAM. 2006a. Background Review Document: *In Vitro* Basal Cytotoxicity Test Methods for Estimating Acute Oral Systemic Toxicity. Research Triangle Park, NC: National Institute for Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_brd.htm

(7) ICCVAM. 2006b. Peer Review Panel Report: The Use of *In Vitro* Basal Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Testing. Research Triangle Park, NC: National Institute for Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_scpeerrev.htm

(8) ICCVAM. 2006c. ICCVAM Test Method Evaluation Report: *In Vitro* Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Tests. Research Triangle Park, NC: National Institute for Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_tmer.htm

(9) ICCVAM. 2001. Report of the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity. NIH Publication No. 01-4499. Research Triangle Park, NC: National Institute for Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/acutetox/inv_cyto_wksp.htm

(10) OECD. 2008. Guideline for Testing of Chemicals, 425, Acute Oral Toxicity – Up-and-Down Procedure. Paris, France:Organisation for Economic Co-operation and Development.

(11) OECD. 2001. Guideline For Testing of Chemicals, 423, Acute Oral Toxicity – Acute Toxic Class Method. Paris, France:Organisation for Economic Co-operation and Development.

(12) Seibert, H., Balls, M., Fentem, J.H., Bianchi, V., Clothier, R.H., Dierickx, P.J., Ekwall B., Garle, J.J., Gómez-Lechón, M.J., Gribaldo, L., Gülden, M., Liebsch, M., Rasmussen, E., Roguet, R., Shrivastava, R., and Walum, E. 1996. Acute toxicity testing *in vitro* and the classification and labelling of chemicals. The report and recommendations of ECVAM Workshop 16. *Alternatives to Laboratory Animals* 24:499-510.

(13) Spielmann H., Genschow E., Liebsch M., and Halle W. 1999. Determination of the starting dose for acute oral toxicity (LD_{50}) testing in the up and down procedure (UDP) from cytotoxicity data. Altern Lab Anim 27:957-966.

(14) Stokes W, Casati S, Strickland J, Paris M. 2008. Neutral Red Uptake Cytotoxicity Tests for Estimating Starting Doses for Acute Oral Toxicity Tests. Current Protocols in Toxicology. May. Suppl. 36: 20.4.1-20.4.20.

(15) UN. 2007. Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Second Revised Edition. [ST/SG/AC.10/30/Rev.2]. New York and Geneva:United Nations. Available: http://www.unece.org/trans/danger/publi/ghs/ghs_rev02/02files_e.html

940 **ANNEX 1**

941 **DEFINITIONS**

942 Coefficient of determination: In linear regression, it denotes the proportion of the variance in Y and X that
943 is shared. Its value ranges between zero and one and it is commonly called "R^2." For example, $R^2 = 0.45$,
944 indicates that 45% of the variance in Y can be explained by the variation in X and that 45% of the
945 variance in X can be explained by the variation in Y.

946 Coefficient of variation: A statistical representation of the precision of a test. It is expressed as a
947 percentage and is calculated as follows: (standard deviation/mean) × 100%

948 Confluence: A state in which cells in culture encounter other cells in the same culture to form a complete
949 sheet of cells (monolayer). Confluence is determined as a percentage of cell coverage of the tissue culture
950 vessel growth surface (e.g., cell monolayer is 80% confluent).

951 Cytotoxicity: The adverse effects resulting from interference with structures and/or processes essential for
952 cell survival, proliferation, and/or function. For most chemicals/substances, toxicity is a consequence of
953 non-specific alterations in "basal cell functions" (i.e., via mitochondria, plasma membrane integrity, etc.),
954 which may then lead to effects on organ-specific functions and/or death of the organism. These effects
955 may involve the integrity of membranes and the cytoskeleton, cellular metabolism, the synthesis and
956 degradation or release of cellular constituents or products, ion regulation, and cell division.

957 Hill function: The IC_{50} values are determined from the concentration-response using a Hill function which
958 is a four-parameter logistic mathematical model relating the concentration of the test substance to the
959 response (typically following a sigmoidal shape).

960 $$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log EC50 - \log X)\text{HillSlope}}}$$

961 where Y=response (i.e., % viability), X is the substance concentration producing the response, Bottom is
962 the minimum response (0% viability, maximum toxicity), Top is the maximum response (maximum
963 viability), EC_{50} is the substance concentration at the response midway between Top and Bottom, and
964 HillSlope describes the slope of the curve. When Top=100% viability and Bottom=0% viability, the EC_{50}
965 is the equal to the IC_{50}.

966 Hill function (rearranged): Some unusual dose-responses do not fit the Hill function well. To obtain a
967 better model fit, the Bottom parameter can be estimated without constraints (i.e., Bottom not necessarily
968 any particular value). However, when Bottom≠0, the EC_{50} reported by the Hill function is not the same as
969 the IC_{50} since the Hill function defines EC_{50} as the point midway between Top and Bottom. Thus, the Hill
970 function calculation using the Prism® software was rearranged to calculate the concentration
971 corresponding to the IC_{50} as follows:

$$\log IC_{50} = \log EC_{50} - \frac{\log\left(\frac{Top - Bottom}{Y - Bottom} - 1\right)}{HillSlope}$$

972

973 where IC_{50} is the concentration producing 50% toxicity, EC_{50} is the concentration producing a response
974 midway between the Top and Bottom responses; Top is the maximum response (maximum survival),
975 Bottom is the minimum response (0% viability, maximum toxicity), Y=50 (i.e., 50% response), and

HillSlope describes the slope of the response. The X from the standard Hill function equation is replaced, in the rearranged Hill function equation, by the IC_{50}.

IC_{50}: Test chemical/substance concentration producing 50% inhibition of the endpoint measured (i.e., cell viability).

LD_{50}: The calculated value of the oral dose that produces lethality in 50% of test animals (rats and mice). The LD_{50} values serve as reference values for the *in vitro* tests.

Neutral red uptake (NRU): Concentration of neutral red dye in the lysosomes of living cells. Altering the cell surface or the lysosomal membrane by a toxicological agent causes lysosomal fragility and other adverse changes that gradually become irreversible. The NRU test method makes it possible to distinguish between viable, damaged, or dead cells because these changes result in decreased uptake and binding of NR measurable by optical density absorption readings in a spectrophotometer.

Optical density (OD_{540}): The absorption (i.e., OD_{540} measurement) of the resulting colored solution (colorimetric endpoint) in the NRU assay measured at 540 nm ±10 nm in a spectrophotometric microtiter plate reader using blanks as a reference.

RC millimole regression: $\log (LD_{50}) = 0.435 \log (IC_{50}) + 0.625$; for estimating an LD_{50} value in mmol/kg (body weight) from an IC_{50} value in mM. Developed using the 347 IC_{50} and oral LD_{50} (282 rat and 65 mouse) values from the RC.

RC rat-only millimole regression: $\log (LD_{50}) = 0.439 \log (IC_{50}) + 0.621$; for estimating an LD_{50} value in mmol/kg (body weight) from an IC_{50} value in mM; developed from the IC_{50} values (in mM) and acute oral LD_{50} values (in mmol/kg) for the 282 substances with rat LD_{50} values in the RC database (Halle 1998, 2003).

RC rat-only weight regression: $\log (LD_{50}) = 0.372 \log (IC_{50}) + 2.024$; for estimating an LD_{50} value in mg/kg (body weight) from an IC_{50} value in μg/mL; developed from the IC_{50} values (in μg/mL) and acute oral LD_{50} values (in mg/kg) for the 282 substances with rat LD_{50} values in the RC database (Halle 1998, 2003).

Solubility: The amount of a test substance that can be dissolved (or thoroughly mixed with) culture medium or solvent. The solubility protocol was based on a U.S. EPA guideline (EPA, 1996) that involves testing for solubility in a particular solvent, beginning at a relatively high concentration and proceeding to successively lower concentrations by adding more solvent as necessary for dissolution. Testing stops when, upon visual observation, the procedure produces a clear solution with no cloudiness or precipitate.

Volatility: Ability of a test chemical/substance to evaporate. A general indicator of excessive volatility in the NRU test methods is the percent difference in the mean OD_{540} values for the two VC columns on the test plate (i.e., excessive volatility contaminates the VC column adjacent to the highest test substance concentration). If the difference is greater than 15%, then excessive chemical/substance volatility can be suspected, especially if the VC adjacent to the highest test concentration had a significantly reduced OD_{540} value. Excessive volatility may be an issue for compounds with a specific gravity of less than 1.

ANNEX 2

PREQUALIFICATION OF NORMAL HUMAN EPIDERMAL KERATINOCYTE (NHK) GROWTH MEDIUM

1. Keratinocyte Basal Medium and the medium supplements supplied by a manufacturer for use with normal human epidermal keratinocytes (NHK) should be prequalified to demonstrate their ability to perform adequately in the NHK NRU assay. The quality control (QC) test data should be obtained from the manufacturer for each potential lot of medium and supplements.

Test System

2. The NHK NRU assay is performed to analyze NHK growth characteristics and the *in vitro* toxicity of SLS, as measured by the IC_{50}, for each NHK medium/supplement combination being tested. Test every combination of medium/supplements expected to be used in subsequent NHK NRU tests.

3. Establish NHK cultures using each medium/supplement combination to be tested, and subculture the cells on three different days into 96-well plates (1 plate per day) for three subsequent SLS cytotoxicity tests using each test medium/supplement combination along with a control medium/supplement (if available) for which performance has been previously established.

Test Methods

4. Establish NHK cultures with cryopreserved cells seeded into individual 25 cm^2 tissue culture flasks using a proven medium/supplement combination (i.e., the control medium) and each test medium/supplement combination.

5. Suspend freshly thawed cells initially into 9 mL of control medium and then add the cell suspension to 25 cm^2 culture flasks containing pre-warmed control or test medium. Use cell seeding densities in flasks (1 flask/density/medium) of 1×10^4, 5×10^3, and 2.5×10^3 cells.

6. Subculture the cells on three different days into 96-well plates for three subsequent NRU tests (three test plates total [one plate per day] for each medium/supplement combination and each control).

7. Subculturing the cells and application of the SLS will follow the procedures in methods in Stokes et al. (2008) in reference to appropriate cell confluency. Cell numbers should be recorded for each flask prior to subculturing to the 96-well plates. Doubling time may be measured as an additional quality assurance check.

Test Procedure

8. Preparation of SLS should follow the main test procedures for testing compounds in keratinocyte routine culture medium. Cells cultured in control medium and in each test medium/supplement combination should be tested in parallel for their sensitivity to SLS.

9. SLS concentrations should be the same or similar to those used previously with control medium/supplements. The SLS concentration range used in an *in vitro* validation study was 0.6 μg/mL – 20.0 μg/mL (ICCVAM, 2006a).

Microscopic Evaluation

10. Changes in morphology of the cells due to cytotoxic effects of the SLS (prior to measurement of NRU) should be recorded. In addition to the general microscopic evaluation of the cell cultures, the following specific observations should be made:

General culture observations

- Rate of proliferation (e.g., rapid, fair, slow)
- Percent confluence (e.g., daily estimate)
- Number of mitotic figures (e.g., average per field)
- Contamination (present/not present)

Cell morphology observations

- Overall appearance (e.g., good, fair, poor)
- Colony formation (e.g., tight/defined, fair, loose/migrating)
- Distribution (e.g., even/uneven)
- Abnormal cells (e.g., enlarged, vacuolated, necrotic, spotted, blebby - [average per field])

Data Analysis and Test Evaluation

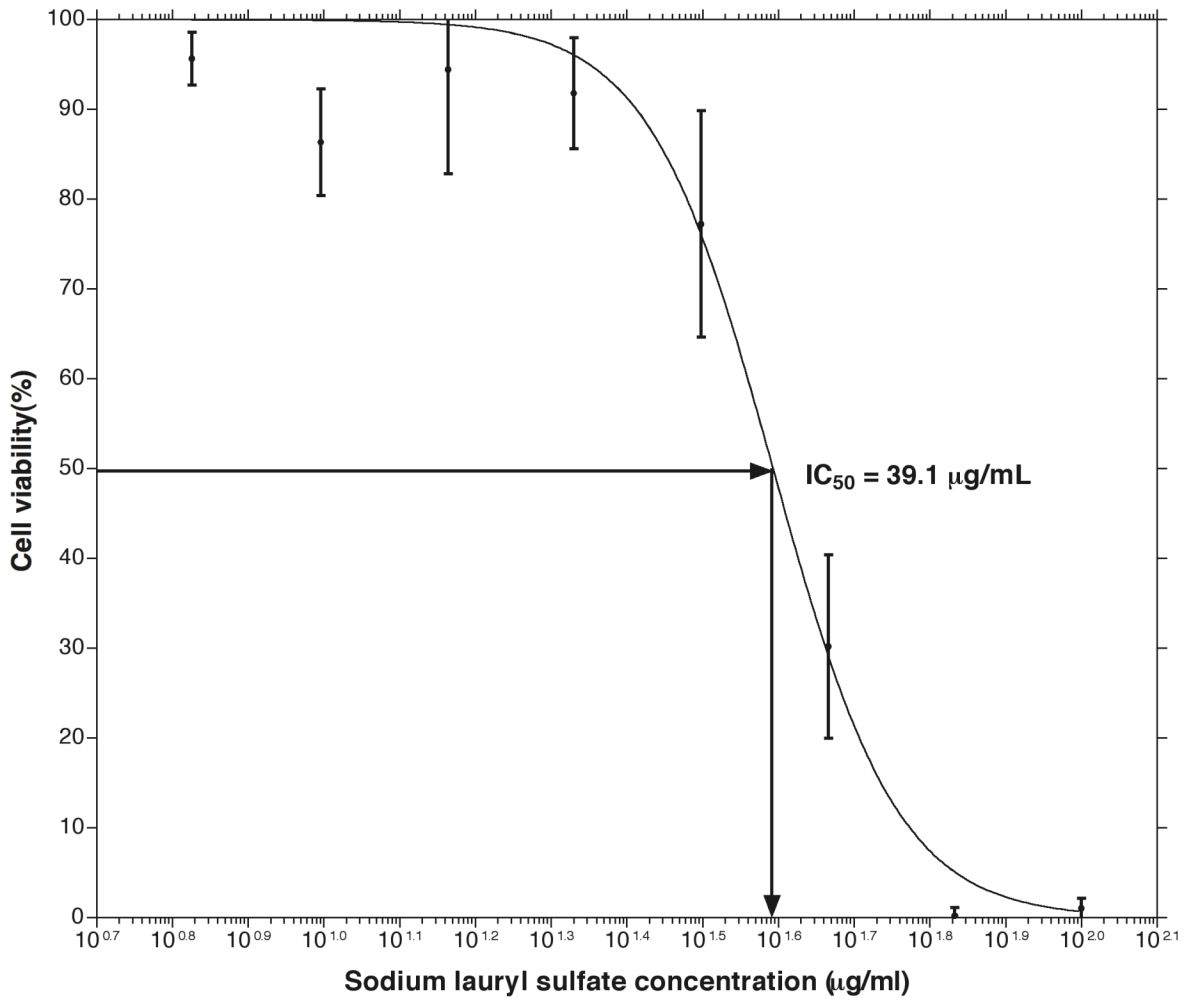
11. See Test Acceptance Criteria (paragraphs 45-48) to determine acceptability of a test plate. Other criteria that should be considered include the following:

- Mean corrected OD_{540} of the VCs. *Note: The target range for corrected mean OD_{540} = 0.248 - 1.123 for the VCs (range = mean OD_{540} ±2.5 standard deviations; mean = 0.685; SD = 0.175; N = 114 [ICCVAM, 2006a]).*
- Cell morphology and confluence of the VCs at the end of the 48-hour treatment.
- Doubling time for NHK cells.

12. Utilize all observed growth characteristics and test results in addition to comparison of results to the media manufacturer's QC data to determine whether the medium/supplements combinations perform adequately.

ANNEX 3

Typical Dose-Response for Sodium Lauryl Sulfate (SLS) in the Neutral Red Uptake Test Using BALB/c 3T3 Mouse Fibroblasts



The points and error bars show the means and standard deviations, respectively, for the percent cell viability response of the six replicate wells at each of the eight concentrations: 6.8, 10, 14.7, 21.5, 31.6, 46.4, 68.1, and 100 μg/mL. The curved line shows the fit of the concentration-response to the Hill function.

ANNEX 4

96-WELL PLATE TEMPLATE

	1	2	3	4	5	6	7	8	9	10	11	12
A	VCb	VCb	C_1b	C_2b	C_3b	C_4b	C_5b	C_6b	C_7b	C_8b	VCb	VCb
B	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
C	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
D	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
E	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
F	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
G	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
H	VCb	VCb	C_1b	C_2b	C_3b	C_4b	C_5b	C_6b	C_7b	C_8b	VCb	VCb

96-Well plate configuration for positive control (PC) and test substance assays.

Rows A through H show the locations of the eight rows of the 96-well plate, while the columns numbered 1 through 12 show the locations of the 12 columns of the 96-well plate.

VC1 and VC2 are the left (VC1) and right (VC2) vehicle control wells, which contain cells, routine culture medium and solvent (if used). VCb wells are VC blanks that contain routine culture medium and solvent [if used], but not cells.

C_1 – C_8 are the eight test substance or PC (sodium lauryl sulfate [SLS]) concentrations. C_1 is the highest concentration and C_8 is the lowest. Each concentration tested has six replicate wells. C_xb are blank wells that contain test substance or PC, but not cells.

ANNEX 5

SOLUBILITY PROTOCOL

SOLUBILITY DETERMINATION OF TEST SUBSTANCES

1. This protocol identifies the solvent that provides the highest soluble concentration of a test substance for uniform availability of the substance to cells in *in vitro* basal cytotoxicity testing.

2. The solubility test procedure is based on attempting to dissolve a test substance in various solvents with increasingly rigorous mixing techniques. The solvents to be used, in the order of preference, are cell culture medium, DMSO, and ETOH. Determination of whether a test substance has dissolved can be based on visual observation using a microscope. A test substance has dissolved if the solution is clear and shows no signs of cloudiness or precipitation.

3. The solubility test procedure is a step-wise tiered procedure to determine the appropriate solvent for use in the test methods. Each tier involves attempting to dissolve the test substance in one or more solvents at test substance concentrations that will yield the same concentration (when dissolved in any solvent) on the cells (with 0.5% [v/v] DMSO or ETOH for those substances not soluble in medium). If the test substance does not dissolve in the solvent, the volume of solvent is increased so as to decrease the test substance concentration by a factor of 10, and then the sequence of mixing procedures are repeated in an attempt to solubilize the substance at the lower concentration. If all solvents for a particular tier are tested simultaneously and a test substance dissolves in more than one solvent, then the choice of solvent follows the culture medium, DMSO, and ETOH hierarchy. If, at any tier, a substance were soluble in medium and DMSO, the choice of solvent would be medium. If the substance were insoluble in medium, but soluble in DMSO and ETOH, the choice of solvent would be DMSO.

Determination of Solubility Using the Step-Wise (Tiered) Procedure

4. *Tier 1*: Weigh 100 mg of the test substance into a glass tube. Add approximately 0.5 mL of medium into the tube to get 200 mg/mL. Mix the solution. If complete solubility is achieved, then additional solubility procedures are not needed.

5. *Tier 2*: If the test substance is insoluble in Tier 1 at 200 mg/mL, then proceed to Tier 2. Weigh 10 mg of the test substance into a glass tube. Add approximately 0.5 mL of medium to get 20 mg/mL. Mix the solution. If complete solubility is achieved, then additional solubility procedures are not needed.

6. *Tier 3*: If the test substance is insoluble in Tier 2 at 20 mg/mL, proceed to Tier 3. Add enough medium, approximately 4.5 mL, to attempt to dissolve the substance at 2 mg/mL by using the sequence of mixing procedures. If the test substance dissolves in medium at 2 mg/mL, no further procedures are necessary. If the test substance does not dissolve in medium, weigh 100 mg test substance in a second glass tube and add approximately 0.5 mL DMSO to get 200 mg/mL and mix the solution. If the test substance does not dissolve in DMSO, weigh 100 mg test substance in another glass tube and add approximately 0.5 mL ETOH to get 200 mg/mL and mix the solution. If the substance is soluble in either solvent, no additional solubility procedures are needed.

7. *Tier 4*: If the substance is insoluble in Test Substance Dilution Medium, DMSO, or ETOH at Tier 3, then continue to Tier 4. Add enough solvent to increase the volume of the three (or four) Tier 2 solutions by 10 and attempt to solubilize again using the sequence of mixing procedures. If the test substance dissolves, no additional solubility procedures are necessary. If the test substance does not dissolve, continue with Tier 5 and, if necessary, Tier 6 using DMSO and ETOH.

8. *Tier 5*: Dilute the Tier 4 samples with DMSO or ETOH to bring the total volume to 50 mL and attempt to solubilize again using the sequence of mixing procedures.

9. *Tier 6*: Weigh two samples of test substance at 10 mg each, add approximately 50 mL DMSO or ETOH for a 200 μg/mL solution, and following the mixing procedures.

Mixing Procedures

10. The following hierarchy of mixing procedures will be followed to dissolve the test substance:

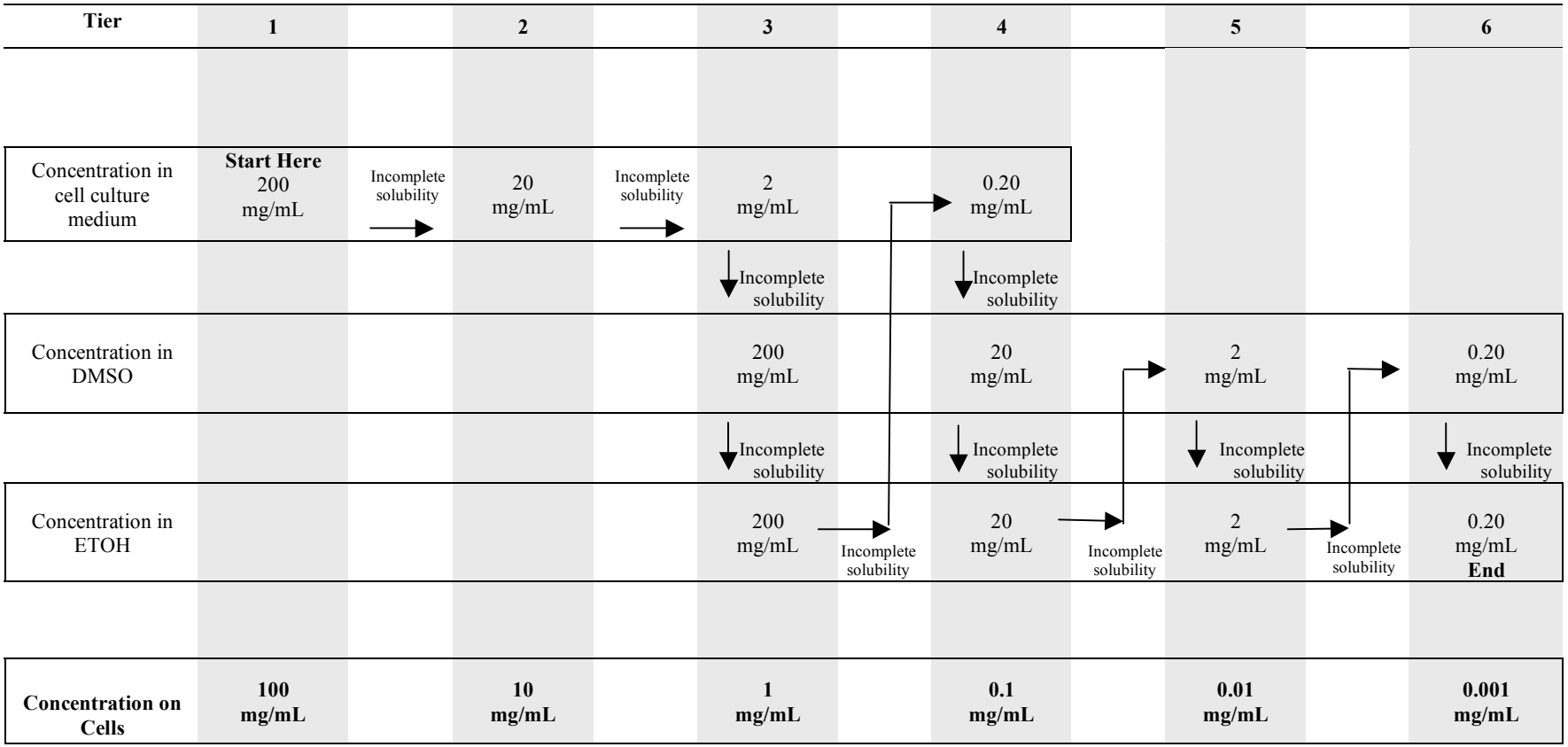
a) Gently mix at room temperature by vortexing for 1 – 2 minutes.

b) If test substance has not dissolved, use waterbath sonication for up to 5 minutes.

c) If test substance is not dissolved after sonication, then warm solution to 37°C for 5 - 60 minutes in a waterbath or in a CO_2 incubator. The solution may be stirred during warming (stirring in a CO_2 incubator will help maintain proper pH).

d) Proceed to Tier 2 (and Tiers 3-6, if necessary and repeat mixing procedures a - b).

Figure 1. Flow Chart for Determination of Test Substance Solubility in Medium, Dimethyl Sulfoxide (DMSO), or Ethanol (ETOH).



Testing starts with 200 mg/mL cell culture medium and proceeds to 0.2 mg/mL in ETOH if the test substance is not completely soluble. Mixing procedures are applied at each concentration step to enhance dissolution. Testing stops at any step during which the test substance achieves solubility.

ANNEX 6

TROUBLESHOOTING

1. The success of a NRU test outcome depends upon achieving adequate cell growth, sufficient cytotoxicity for the calculation of an IC_{50} value, the absence of neutral red crystals, and a good fit of the concentration-response data to the Hill function. Cells should be in the exponential phase of growth during chemical/substance exposure. Control OD_{540} values should typically be at least 0.3, although lower OD_{540} measurements can be justified if the cells look healthy and the response to SLS is adequate. If neither of these conditions is met, suspect mycoplasma (or other; e.g., bacterial, fungal) contamination, inadequate environmental conditions (temperature, CO_2, humidity), cell culture medium, or cell culture medium components (i.e., serum for the 3T3 or growth factors for the NHK). Although 100% confluence at the end of the exposure period is satisfactory for the 3T3 cells, it is undesirable for the NHK cells. Confluent NHK cells produce growth factors that inhibit growth and promote differentiation.

2. Solubility is often the limiting factor in achieving sufficient cytotoxicity for the calculation of an IC_{50} value, especially for relatively nontoxic test substances. Insoluble substances may produce a precipitate or a film in the stock solution or in the cell culture wells. Solvents other than those recommended in this protocol may be used if the concentration used does not produce cytotoxicity. Additional procedures such as stirring or heating for longer periods may also increase test substance solubility. Users should be aware that inadequate toxicity upon exposure to volatile substances might, in fact, be an artifact of the "airborne" substance escaping the wells. A reduction in the viability of the VC cultures adjacent to the highest concentration of a test substance may suggest that this substance has volatilized (see VC1 in ANNEX 5). However, adequate cytotoxicity for some volatile agents is achievable with the use of plastic film sealers to retain the vapors and minimize contamination of neighboring VC wells.

3. NR dye crystals interfere with OD_{540} measurements. Blank OD_{540} values may increase from the typical 0.05 to approximately 0.10 or higher. Preparation and maintenance of the NR dye solution is a key factor in minimizing crystal formation. Therefore, the NR dye solution should be made fresh, filtered, and maintained at 37ºC prior to application to the cells.

4. The calculation of an appropriate IC_{50} value depends upon the fit of the concentration-response data to the Hill function. Toxicants that are specific for acting at a single phase of the cell cycle may yield a concentration-response in which percent viability oscillates greatly around 50% with the increasing log doses of the range finder test. In these situations, the main test should focus on the lowest concentrations that produce 50% reduction in viability. Concentration-responses, for which the percent viability plateaus with increasing concentration, rather than decreasing to 0%, tend to fit the Hill function poorly (i.e., $R^2 <$ 0.9). The fit is generally improved by allowing the Hill function to fit the Bottom parameter of the Hill function rather than by constraining it to 0% viability. Then, however, the EC_{50} of the standard Hill function will not be equivalent to the concentration that reduces viability by 50%. The Hill function calculation should be rearranged to calculate the IC_{50} as follows:

$$\log IC_{50} = \log EC_{50} - \frac{\log\left(\frac{Top - Bottom}{Y - Bottom} - 1\right)}{HillSlope}$$

where IC_{50} is the concentration producing 50% toxicity, EC_{50} is the concentration producing a response midway between the Top and Bottom responses; Top is the maximum percent viability, Bottom is the minimum viability (maximum toxicity), Y=50 (i.e., 50% response), and HillSlope describes the slope of

1199 the response. The X from the standard Hill function equation is replaced, in the rearranged Hill function
1200 equation, by the IC_{50}.

1201 5. The prediction of the rat oral LD_{50} values and the determination of starting doses for acute oral
1202 toxicity tests by the *in vitro* NRU methods is expected to be poor for substances with mechanisms of
1203 toxicity that are not active in the 3T3 or NHK cells. Such toxic mechanisms include specific, receptor-
1204 mediated actions on the central nervous system or the heart (ICCVAM, 2006a).

1205

1206

ANNEX 7

EXAMPLES FOR ESTIMATION OF STARTING DOSES FOR ACUTE ORAL SYSTEMIC TOXICITY TESTS

(see **Determination of the Starting Doses for Acute Oral Systemic Toxicity Tests** – paragraphs 57-60)

EXAMPLE FOR mM IC_{50} VALUE

Acetylsalicylic Acid (MW 180.20)

3T3 NRU IC_{50} = 3.750 mM

log LD_{50} (mmol/kg) = 0.439 log IC_{50} (mM) + 0.621 (ICCVAM, 2006a).

log LD_{50} (mmol/kg) = (0.439 x 0.574 mM) + 0.621

log LD_{50} (mmol/kg) = 0.873

LD_{50} = 7.464 mmol/kg

Estimated LD_{50} = 7.464 mmol/kg x 180.20 mg/mmol

Estimated LD_{50} = 1346 mg/kg

UDP Starting Dose

Default doses: 1.75, 5.5, 17.5, 55, 175, 550, and 2000 mg/kg (limit test of 2000 mg/kg)
1.75, 5.5, 17.5, 55, 175, 550, 1750, and 5000 mg/kg (limit test of 5000 mg/kg)

Estimated LD_{50} = 1346 mg/kg; Starting dose = 550 mg/kg, one default dose below the estimating LD_{50}.

ATC Starting Dose

Default doses: 5, 50, 300, and 2000 mg/kg (limit test of 2000 mg/kg)
5, 50, 300, 2000, and 5000 mg/kg (limit test of 5000 mg/kg)

Estimated LD_{50} = 1346 mg/kg; Starting dose = 300 mg/kg, one default dose below the estimating LD_{50}.

EXAMPLE FOR µg/mL IC_{50} VALUE

Acetylsalicylic Acid (MW 180.20)

3T3 NRU IC_{50} = 676 µg/mL

log LD_{50} (mg/kg) = 0.372 log IC_{50} (µg/mL) + 2.024 (ICCVAM, 2006a)

log LD_{50} (mg/kg) = (0.372 x 2.83) + 2.024

log LD_{50} (mg/kg) = 3.077

LD_{50} = 1194 mg/kg

UDP Starting Dose

Default doses: 1.75, 5.5, 17.5, 55, 175, 550, and 2000 mg/kg (limit test of 2000 mg/kg)
1.75, 5.5, 17.5, 55, 175, 550, 1750, and 5000 mg/kg (limit test of 5000 mg/kg)

Estimated LD_{50} = 1194 mg/kg; Starting dose = 550 mg/kg, one default dose below the estimating LD_{50}.

ATC Starting Dose

Default doses: 5, 50, 300, or 2000 mg/kg (limit test of 2000 mg/kg)
5, 50, 300, 2000, or 5000 mg/kg (limit test of 5000 mg/kg)

Estimated LD_{50} = 1194 mg/kg; Starting dose = 300 mg/kg, one default dose below the estimating LD_{50}.

Table 1 Linear Regression Analyses to Improve the Prediction of Rodent Acute Oral LD_{50} Values from *In Vitro* NRU IC_{50} Using the RC Database[1]

Data Used	Slope	Intercept	R^2
347 RC substances (282 rat and 65 mouse LD_{50} values) – millimole units[2]	0.435	0.625	0.452[3]
282 RC substances with rat LD_{50} data – millimole units[2]	0.439	0.621	0.452
282 RC substances with rat LD_{50} data – weight units[4]	0.372	2.024	0.325

Abbreviations: NRU=Neutral red uptake; RC=Registry of Cytotoxicity; R^2=Coefficient of determination.

[1]Slopes of all regressions were significantly different ($p < 0.05$) from zero at $p < 0.0001$.

[2]IC_{50} in mM; LD_{50} in mmol/kg.

[3]Calculated from RC data (i.e., not reported by Halle [1998, 2003]).

[4]IC_{50} in μg/mL; LD_{50} in mg/kg.